A cross-species and model comparison of the acute toxicity of nanoparticles used in the pigment and ink industries

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Abstract

A major user of nanoparticles (NPs) is the pigment and ink industry, where NPs are incorporated into numerous products (e.g. paints, food, plastics, printers, personal care products, and construction materials). Assessment of NP toxicity requires potential impacts on human health and the environment to be evaluated. In this study, we examined the toxicity of a range of NPs, of varied physico-chemical properties, used in the pigment and ink industries including silver (Ag), iron oxide (Fe_2O_3), titanium dioxide (TiO_2), aluminium oxide (Al_2O_3), zinc oxide (ZnO), cobalt aluminium oxide ($CoAl_2O_4$) and cadmium selenide / zinc sulphide (CdSe/ZnS) quantum dots (QDs). Acute toxicity exerted by this NP panel to mammalian cells *in vitro* (macrophages, hepatocytes and alveolar epithelial cells) and aquatic environmental organisms (*Raphidocelis subcapitata Daphnia magna, Lumbriculus variegatus*) was investigated. For mammalian cells, cytotoxicity was assessed 24 h post exposure, at concentrations ranging from 1-125µg/ml using the LDH and WST-1 assays. The aquatic toxicity of the NP panel was assessed according to OECD protocols (201, 202, 315), up to 96 h post exposure. Rats were exposed to selected NPs via intratracheal instillation (62µg) and the pulmonary inflammatory response quantified 24h post exposure. This cross-species comparison revealed that Ag, QDs and ZnO NPs were consistently more toxic than the other NPs tested. By looking across mammalian and aquatic ecotoxicological models we obtained a better understanding of the sensitivity of each model, and thus which models should be prioritised for selection in the future when assessing the

mammalian and ecotoxicity of NPs, and in particular when screening the toxicity of a panel of NPs. We recommend that macrophage and daphnia models are prioritised when assessing the mammalian toxicity and ecotoxicity of NPs, respectively, due to their increased sensitivity, compared to the other models tested. Of interest is that the *in vitro* and invertebrate models used were able to predict the toxic potency of the NPs in rodents, and thus our approach has the potential to enhance the implementation of the 3Rs principles in nanotoxicology and reduce reliance on rodent testing when assessing NP safety. By identifying hazardous NPs the data obtained from this study can feed into the selection of (low toxicity) NPs to use in products and will also contribute to the safe design of future generations of NPs used by the pigment and ink industries.

1. Introduction

A major user of nanoparticles (NPs) is the pigment and ink industry, where NPs are used in numerous products such as paints, food, plastics, paper, printers, dyes, personal care products (e.g. toothpaste, cosmetics), ceramics, and construction materials (e.g. Weir *et al*., 2012). A range of different NPs are exploited by these industries, for example, TiO_2 NPs are commonly used as white pigments in food, personal care products and paints (Weir *et al*., 2012), whilst iron oxide NPs can be used in the building and paper industries (Montes-Hernandez *et al*., 2006). The physico-chemical properties of NPs (e.g. particle size, chemical composition, morphology and surface charge) are able to influence their biological behaviour. A huge diversity of NPs are used by the pigment and inks industries, hence improving our understanding of the relationship between NP physico-chemical properties and their hazard potential will be critical to the safe and responsible development of nanotechnology. This includes decision making (e.g. selection of (low toxicity) NPs to use in products/applications), informing safety by design as well as supporting the development of evidence based legislation and risk management measures to protect human health and the environment from any potential risks of NPs.

The use of NPs by the pigment and ink industry means that humans may be exposed to these materials via inhalation, ingestion (via hand to mouth contact), and dermal routes in occupational, consumer and environmental settings during their production, use and disposal. Our study was focused on assessment of the hazards posed by NPs to human health in an occupational setting, and investigated the response of lung epithelial cells, macrophages and hepatocytes to NPs *in vitro*.

Assessment of the response of the lung is critical within NP safety assessments as exposure via inhalation is anticipated to be one of the primary routes of human exposure in an occupational setting. It is established that NPs can deposit in the alveolar region of the lung following pulmonary exposure (Oberdorster *et al*., 2002., Semmler-Behnke *et al*., 2008) and from there are able to translocate to other areas of the body. Thus, many studies have assessed the response of alveolar epithelial cells, and in particular the human A549 cell line, to NPs of varied physico-chemical characteristics such as ufCB, Ag, TiO2 (e.g. Geiser *et al*., 2005). Accordingly, we selected the A549 cell line to evaluate the toxicity of NPs used by the pigment and ink industries to the lung.

NPs have been observed to cross the epithelial barrier of the lung (e.g. Oberdorster *et al*., 2002), to reach the blood circulation. Translocation of NPs from the lung, and their accumulation NPs in secondary target sites suggests that there may be widely distributed toxic effects (Oberdorster *et al*., 2005). A major site of NP sequestration after intravenous injection (Ogawara *et al.*, 1999; Semmler-Behnke *et al*., 2008), pulmonary exposure (Nemmar *et al.*, 2002, Takenaka *et al.*, 2001, Oberdorster *et al*., 2002, Semmler et al, 2004) or ingestion (Schleh *et al*., 2012, Jani et al 1990) is the liver. The liver may therefore be a prime target organ for NPs, regardless of the route of exposure, and thus investigation of the hepatic response to NPs is relevant when performing safety assessments for NPs. *In vitro* studies have primarily assessed the response of hepatocytes when investigating the hepatotoxicity of NPs as hepatocytes represent the main cell population in the liver. Of interest is that the response of hepatocyte cell lines (e.g. C3A) has been found to be comparable to that of primary rat or human cells when NP toxicity has been assessed previously (e.g. Johnston et al., 2010, Kermanizadeh et al 2013). Furthermore, the toxicity exhibited by Ag NPs to the liver *in vivo* has been observed to be similar to the response observed *in vitro* (C3A

cell line) (Gaiser *et al*., 2014), which promotes the use of non-rodent, alternative models when assessing NP toxicity. Accordingly, the C3A hepatocyte cell line was selected for investigation of NP toxicity in this study.

Macrophages represent the major cell type of the immune system responsible for the clearance of NPs from the lungs and other tissues (e.g. liver) (Geiser *et al*., 2008, Semmler-Behnke *et al*., 2007, Ogawara *et al*., 1999). Similarly, phagocytosis of NPs by macrophages *in vitro* has been observed for many NP types (e.g. Gehr et al., 2011), Furthermore, Kupffer cells (resident liver macrophages) have been observed to play a central role in the liver's response to Ag NPs *in vivo,* following intravenous administration (Kermanizadeh *et al*., 2014). Interestingly, it has been observed that macrophage responses to NPs *in vitro* can predict the pulmonary toxicity of NPs in rodents following inhalation (e.g. Weimann *et al*., 2016). Thus assessment of the macrophage response is prudent when investigating the response of the lung and liver to NPs. A huge variety of cell types have been used to investigate to the response of macrophages to NPs *in vitro*, including cell lines (e.g. THP-1, J774, MM6, RAW264.7, NR8383), primary human rat or mouse macrophages (derived from blood, the lungs or the peritoneum). We selected the murine J774 macrophage-like cell line as we have previously demonstrated that this cell type can provide a comparable response to that of primary macrophages (e.g. Brown *et al*., 2004).

Comparison of NP toxicity across the different cell types was assessed via investigation of the impact of NPs on cell viability. This approach enabled ranking of the toxicity of a panel of different NPs across cell models, to identify differences in cell sensitivity and to rank NP toxicity. We compared two assays which measure cell viability/cytotoxicity via different approaches; the WST-1 assay which assesses mitochondrial function as an indicator of cell viability, and the lactate dehydrogenase (LDH) assay which measures release of LDH from cells to assess plasma membrane integrity, and is indicative of cell death. The sensitivity of each cytotoxicity/viability assay was compared in order to identify those potentially useful for identifying hazardous materials when screening NP toxicity using *in vitro* models in the future.

In vitro cell based models, representing different target sites, are commonly used to screen NP toxicity in order to decrease the cost and increase the efficiency of testing,

and to better align toxicology testing with the 3Rs principles of scientific research (replacement, refinement and reduction of animal use). However it is necessary to consider whether *in vitro* models are able to predict the *in vivo* response. An infiltration of neutrophils into the exposure site (e.g. lung) is commonly used as an indicator of the acute toxicity of NPs *in vivo* (e.g. Gosens *et al*., 2015, Landsiedel *et al.,* 2014, Poland *et al*., 2008, Brown *et al*., 2001). Therefore, we assessed the ability of selected NPs to stimulate an acute pulmonary inflammatory response in rats following intratracheal instillation in our study. The toxic potency of the NPs observed *in vivo* will be compared to that observed *in vitro* in order to identify if *in vitro* models provide a good prediction of NP toxicity.

The production, use and disposal of NPs are likely to lead to their release into the environment (e.g. via wastewater) (Nowack *et al*., 2012). In parallel to assessing the impacts of NPs on human health it is therefore essential to evaluate the ecotoxicity of NPs. Measurement and modelling studies have analysed and predicted the release levels and fate of NPs into different environmental compartments (e.g. Mueller and Nowack, 2008, Gottshalk *et al*., 2009, Johnson *et al*., 2011, reviewed in Gottschalk, Sun and Nowack, 2013). Evaluation of the aquatic (freshwater and marine) and terrestrial toxicity of NPs is typically evaluated using model environmental organisms, following OECD protocols.

R. subcapitata, D.magna, and *L.variegatus* were selected to assess NP toxicity to aquatic (freshwater) organisms as these have been commonly used to assess the aquatic toxicity of chemicals and NPs previously (e.g. O'Rourke *et al*., 2015, Sohn *et al*., 2015, Khan *et al*., 2015, Li *et al*., 2014, Wang *et al*., 2014). *Raphidocelis subcapitata* is a freshwater microalga, and toxicity to this organism is typically assessed via assessment of growth rate inhibition (via measurement of optical density). (van Hoecke *et al*., 2008). *Lumbriculus variegatus* (California blackworm), is a freshwater dwelling oligochaete which is widespread throughout Europe and North America. It is common in shallow waters, and can burrow into the sediment. *L. variegatus* is often used as a test organism for toxicants applied in water or via sediment (e.g. Pakarinen *et al*., 2011). We tested acute toxicity of NPs to *L. variegatus* via the water column without addition of sediment to have a simple model in which

NPs are easily quantifiable and detectable and to facilitate characterisation of the NPs. Toxicity to this organism is typically assessed via investigation of mortality and behaviour (Rajata *et al*., 2016). *Daphnia magna* are crustaceans that reside in the water column, and toxicity to this organism is typically assessed via investigation of immobility, and impacts on reproduction (OECD Guidelines 1984).

The panel of NPs selected for investigation in this study were; silver (Ag), iron oxide (Fe_2O_3), titanium dioxide (TiO_2), aluminium oxide (Al_2O_3), zinc oxide (ZnO), cobalt aluminium oxide ($CoAl_2O_4$) and cadmium selenide / zinc sulphide (CdSe/ZnS) quantum dots (QDs). The aim of the study was to perform a cross species comparison of the toxicity of this panel of NPs to identify the sensitivity of different mammalian (cell lines and rodents) and environmental models to NPs. In addition, the obtained data were used to compare and rank NP toxicity in order to identify hazardous NPs, whose surface will be modified with the aim of reducing their toxicity. The toxicity of these modified NPs will be investigated in follow on studies, and if the surface modifications successfully reduce NP toxicity, the information will be used to promote the use of low toxicity NPs in products, and to inform the safe design of NPs in the future. A comparison of the toxicity of a panel of NPs across *in vitro* (cell), algal, invertebrate and rodent models is rarely performed. It is therefore envisioned that this study will contribute to the development of an intelligent testing strategy for assessment of NP hazard which promotes the use of non-rodent models. More specifically, by identifying which test systems are most sensitive to NP toxicity we have recommended which models should be prioritised for selection when assessing NP hazard in the future. By comparing the response of *in vitro*, algal, invertebrate and rodent models we will identify opportunities to promote the use of alternative models when assessing NP toxicity to reduce the burden placed on rodent testing in the future. These models will be useful when screening the toxicity of a range of NPs to rank their relative toxicity for the purpose of selecting NPs to prioritise either for use (e.g. low toxicity materials) or for further and more in-depth hazard testing. This testing strategy would be applicable to 'as produced' NPs, but could also be considered for key life-cycle stages of NPs where exposure is demonstrated or expected to be significant. In addition, the models may also be useful in safety by design decision making in order to compare the effectiveness of a range of NP modifications on their hazard. However, we acknowledge that the final choice of

model selected for hazard testing will also be informed by the likely exposure scenario and route of exposure.

2. Materials and Methods

2.1 Particles and particle characterisation

The NPs used in this study, the source of these NPs, and supplier information on NP size are listed in supplementary data Table 1.

2.1.1 Transmission Electron Microscopy (TEM)

Transmission electron microscopy (TEM) analysis was carried out on a Philips CM200 (LaB_6) microscope operating at an acceleration voltage of 200 kV. Samples were prepared by depositing 10µl of NPs suspension (2mg/ml in sterile water and sonicated for five minutes) of each sample on an amorphous carbon film-coated copper grid. Grids were allowed to dry completely before viewing.

2.1.2 Dynamic Light Scattering (DLS)

Size distribution and zeta potentials of the NPs were determined using Dynamic Light Scattering (DLS) (Nanozetasizer, Malvern). The analysis was performed at 25 °C using samples appropriately dispersed and diluted with ddH_2O to a final concentration of 50µg/ml. The suspensions were sonicated for 15 min before the measurements were taken.

2.2 Cell culture

2.2.1 Human A549 Cells

Human A549 epithelial cells were grown in DMEM medium (Life Technologies) containing 10% FCS (Life Technologies), 1% non-essential amino acids (Sigma), sodium pyruvate (1mM, Life Technologies), L-glutamine (2mMLife Technologies), penicillin (100U/ml, Sigma) and streptomycin (100µg/ml, Sigma) (termed complete A549 medium). Cells were removed from culture with trypsin and cells were seeded into 96-well plates at a concentration of $2x10^5$ cells/ml (100µl/well).

2.2.2 J774 Cells

The mouse macrophage cell line J774.A1 was cultured in RPMI medium containing 10% FCS (Life Technologies, final concentration), 1% non-essential amino acids (Sigma), sodium pyruvate (1mM, Life Technologies,), L-glutamine (2mM Life Technologies), penicillin (100U/ml, Sigma,) and streptomycin (100µg/ml, Sigma) (termed complete J774 medium). Cells were seeded at a concentration of $5x10^5$ /ml into 96 well plates (100ul/well).

2.2.3 C3A Cells

The human C3A hepatocyte cell line was cultured in MEM medium containing 10% FCS (Life Technologies, final concentration), 1% non-essential amino acids (Sigma), sodium pyruvate (1mM, Life Technologies), L-glutamine (2mM, Life Technologies), penicillin (100U/ml, Sigma,) and streptomycin (100µg/ml, Sigma (termed complete C3A medium). Cells were removed from culture by treatment with trypsin (2.5mg/ml), trypan blue (0.4% in saline) was used to assess cell viability and cells were seeded into 96-well plates at a concentration of $2x10^5$ cells/ml (100µl/well). Plates were incubated for 24 hours at 37^0C.

2.3 Particle dispersion: mammalian cell toxicology testing

NPs were dispersed in 2% heat inactivated foetal bovine serum (FBS), in water, at a concentration of 2mg/ml and sonicated for 16 minutes, 400W power, then placed on ice. The NP suspensions were then diluted in appropriate complete medium within 15 minutes of initial dispersion.

2.3.1 Cell treatment

Following seeding into a 96 well plates cells were incubated at 37^0C for 24 hours and then washed with medium. NPs were prepared as described above and diluted in cell culture medium at concentrations ranging from 0.16µg/cm^2 to 320µg/cm^2 (0.48-1000µg/ml). The concentration range for Ag NPs was 0.015 to 15.6µg/cm^2 (0.024-50µg/ml). This concentration range was chosen due to the high toxicity of this NP identified in pilot studies. A positive control consisting of 0.1% triton in medium was included. A negative control consisting of complete medium was included for each cell type. To investigate the contribution of soluble metal ions leached from the NPs,

solutions of Zn and Ag ions were prepared from zinc chloride and silver nitrate salts. The number of mmols of Zn and Ag contained in 1mg of each compound was determined from the molecular weight. Solutions of salts of Zn and Ag giving the same concentrations used for the Zn and Ag particle treatments were prepared in culture medium. One hundred microliters of each treatment was added to cells in triplicate, and incubated for 24 hours at 37^0C.

2.3.2 WST-1 Assay

After incubation, the supernatant from each well was removed (and stored at -80^0C until required for the LDH assay (see below)) and replaced with culture medium containing WST-1 reagent (Roche). Cells were incubated for 30 minutes at 37°C and then centrifuged at 250g for 2 minutes, the supernatants removed and transferred to a new 96 well plate and read using a multiwell plate reader at a wavelength of 450nm. Data were expressed as the percentage viability of treated cells compared with the negative control.

In addition to cell treatments, a series of cell free, particle only controls were set up at the same concentrations, but instead of culture medium alone, the NPs were suspended in culture medium containing WST-1 reagent. This control served to detect any particle interference with the assay reagent.

2.3.3 LDH estimations

Sodium pyruvate (Sigma) (0.75mM in water), 50µl, containing 1 mg/ml NADH (Sigma) were pipetted into each well of a 96 well plate and incubated at 37 ^{0}C for 5 minutes. A series of standards were prepared to give a range of dilutions representing 0-2000 LDH Units/ml. Ten microlitres of cell supernatants were added to the wells in triplicate groups and thoroughly mixed. The plates were incubated for 30 minutes at 37^0C. Fifty microlitres of 2,4-dinitrophenylhydrazine (Sigma) solution dissolved in 1M HCl (100µg/ml) were added to each well and incubated at room temperature for 20 minutes. NaOH (4M, 50ul) was then added to each well, mixed and allowed to stand for 5 minutes. The absorbance was read at 540 nm on an automatic plate reader. The LDH content of the supernatants was expressed as a percentage of the 100% triton control.

2.4 *In vivo* Studies

2.4.1 Particle instillations

The particles for this study were selected on the basis of their toxicity as determined in the *in vitro* experiments (above). These particles were: Ag, ZnO and QDots, as well as a relatively less toxic particle type, $CoAl_2O_3$. The particles were dispersed according to the procedure we adopted for the *in vitro* experiments (above).

Male Sprague Dawley rats approximately (3 months old, 250-300g) were used throughout and obtained from the University of Edinburgh and housed in the animal facility at Edinburgh Napier University. Ethical approval was obtained from Edinburgh Napier University ethics committee prior to commencement of the experiments. Animals were allowed free access to food and water throughout the study. Animals were anaesthetised with isoflurane, cannulated intratracheally using a laryngoscope and 0.5ml of each particle type at a concentration of 125µg/ml suspended in saline instilled into the lungs (giving a dose of 62.5µg of particles per animal). All animals were conscious within minutes of this procedure and suffered no ill effects. The animals were sacrificed 24 hours post exposure.

We previously observed that *in vitro* toxicity experiments highlighted a degree of toxicity which could be accounted for by the leaching or dissolution of metal ions from Ag and ZnO particles. Therefore, in addition to the instillation of particles, aqueous solutions of Ag and Zn salts were prepared and these solutions instilled into the rat lungs. Solutions were prepared from silver nitrate and zinc chloride salts ($AgNO_3$ and $ZnCl_2$) at a metal ion concentration which was equivalent to the amount of metal contained in 62.5µg of particles (the dose which was instilled into each animal) assuming that all of the Ag and Zn ions leached from the particles.

2.4.2 Bronchoalveolar Lavage

Rats were sacrificed with a single i.p. injection of Pentobarbitone, the lungs cannulated and removed and lavaged with 4 x 8ml volumes of sterile saline. The lavage fluid was centrifuged at 850g for 2 minutes at 4^{o}C, the supernatant removed and the cell pellet resuspended in 1ml PBS. Cell pellets from each animal were kept separate. Differential cell counts were performed. Three hundred cells per slide were counted and the results expressed as the total number of neutrophils in the lung lavage.

2.5 Ecotoxicology Studies

2.5.1 Nanoparticle Preparation for ecotoxicology studies

Nanoparticle stock suspensions were prepared at a concentration of 500mg/L by bath ultrasonication of a stock in deionised water for 16 minutes, with the container being inverted halfway through the sonication process. For the quantum dots, 5 mg/l of Suwanee River Humic Acid was added to the deionised water prior to sonication, to facilitate dispersion. Stocks were then diluted in the appropriate medium.

2.5.2 D. magna

D. magna organisms were held in culture in Elendt M7 medium (OECD Guidelines for testing of chemicals 202, 1984). This medium was prepared as per OECD guideline 202 by adding the required analytical grade chemicals to Milli-Q grade matrix water. The animals were from a GG4 clone of D. magna Straus, 1820. Animals were cultured under static conditions in 2L beakers containing 1.6L of culture medium. The stock, as well as the experimental animals, were fed the required quantities of *Chlorella vulgaris* daily (or double dosing, if not fed daily), following OECD guidelines.

D. magna were exposed to NPs as neonates (<24 h old). Elendt M7 OECD medium without EDTA was used in these experiments (constituents outlined in OECD Guideline for testing of chemicals 211, 1998). Organisms were added to vials containing medium (5 neonates per vial, in a total volume of 20 ml), and treated with nanoparticles at the following concentrations: ZnO 0.001 – 2mg/l; QDots 0.01 – 100mg/l and Ag – 0.001 – 5mg/l concentrations. This was done in order to avoid contamination of the neonate holding vessel and to avoid trapping neonates on the surface of the suspension by trying to avoid immersing the pipette in the suspensions.

At timepoints of 24 and 48 h after the start of the exposure, dead and immobilised neonates were counted.

2.5.3 L. variegatus

Worms were cultured according to a protocol adapted from the OECD guideline 225 (2007) in the absence of sediment (reconstituted hard water, see supplementary material table 4) in 2 x 10L aquarium tanks in a temperature controlled room at 20 ± 2°C with a light regime of 16:8 hours at 100 - 500 lux. Worms were synchronised by cutting through the middle, and left to regenerate for 10 days prior to exposure to ensure a homogenous population. At the start of the experiment, worms were randomly selected and placed in individual vials containing 20 ml of OECD reconstituted water with or without nanoparticles at the following concentrations: ZnO and QDots 0.001 – 100mg/l; Ag 0.0001 – 5mg/l.

2.5.4 R. subcapitata

Algal tests followed OECD201 protocol except for the use of Jaworski's medium for the exposures (see supplementary material table 5 for components of this medium). Algae were treated with the panel of particles at the following concentrations: ZnO0.01 – 100mg/l; QDots 0.01 – 100mg/l; Ag 0.001-5mg/l; Al_2O_3 0.0 – 1000mg/l; Fe_2O_3 0.0 – 1000mg/l; $CoAl_2O_4$ 0.1 – 1000mg/l and TiO_2 0.0 – 1000mg/l.

Samples were taken at 0, 24, 48 and 72 h following exposure, and the optical density at 685 nm as well as chlorophyll content following acetone extraction (Kalman *et al*., 2015) were measured, and normalised to the corresponding cell numbers. Growth inhibition was calculated in relation to the control for the equivalent time point.

A 100% growth rate inhibition describes stagnation of the population, and an inhibition >100% describes a decrease in the algal cell population, or algal cell death.

2.6 Statistical analysis

Data from all experiments were analysed after checking for normality using the Minitab statistical package using a general linear model with subsequent analysis of variance and Tukey's test. Where necessary, data were transformed using the square root or natural logarithm and the conditions for normality further checked. All analyses were

performed using 'normal' data. Significance was set at 5%. LC_{50} and LC_{20} values were calculated using a four parameter logistic curve with Prism software.

3. Results

3.1 Characterisation

DLS was used to measure the hydrodynamic size of the NP suspensions in water (Supplementary data Table 2). The average hydrodynamic size of Ag NPs and QDots was 29.57±4.83 nm and 36.33±7.26 nm, respectively, suggesting that these NPs were relatively monodispersed. The remaining particle types were agglomerated, with the size of agglomerates ranging from ~220-1500nm, depending on the NP under investigation (Supplementary data Table 2). The zeta potentials of all NP types in water were negatively charged.

A summary of particle characteristics (hydrodynamic diameter and zeta potential), when dispersed in biological media are shown in supplementary data Table 3. Assessment of NP properties in biological media relevant to the *in vitro* and ecotoxicology experiments performed in this study required the preparation of 7 different NPs in 6 different types of media. The data presented in supplementary data Table 3 refer to particles suspended in MEM medium supplemented with 10% FCS (C3A complete medium). The data presented show that the hydrodynamic diameter of Al_2O_3, Ag and QDots were in the nano range (<100nm). TiO_2, ZnO, $CoAl_2O_4$and Fe_2O_3 NPs agglomerated in biological medium. After 24 hours, all of the particle types were agglomerated in culture medium to a greater extent at 0h, with the exception of the Ag NPs. Of interest was the observation that ZnO NPs appeared to be less agglomerated after 24 hours.

TEM was used to visualise NP size and morphology in water (Figure 1). ZnO NPs were polydispersed, with a broad range of shapes (e.g. prisms, bullet-shaped particles, and spheres). Fe_2O_3 NPs were also very polydispersed in size and shape (round to polyhedral), with particles ranging in size from 15 to 90 nm. TiO_2 NPs were highly aggregated NPs with an elongated shape. Ag NPs had various shapes (round to polyhedral). QDs were small in size (average size of 3.6nm) and had a uniform size

and shape. Al_2O_3 NPs were agglomerated, and their shape and size was not clearly distinguishable; while $CoAl_2O_4$ presented round shape morphology.

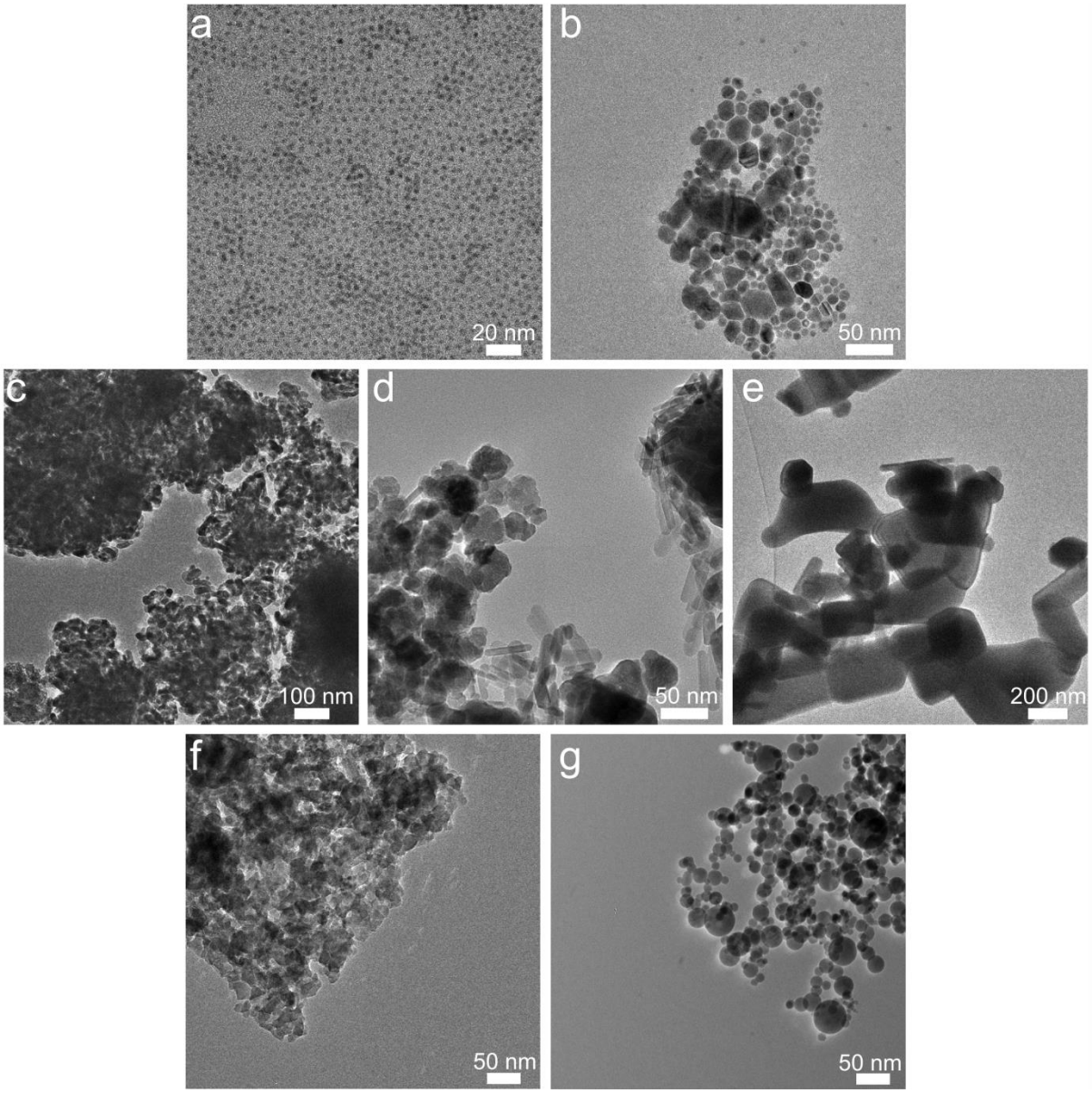

Figure 1. Representative TEM images of (a) QDs, (b) Ag, (c) TiO_2, (d) Fe_2O_3, (e) ZnO, (f) Al_2O_3 and (g) $CoAl_2O_4$ nanoparticles used in this study. See text for sample preparation.

3.2 NP cytotoxicity

3.2.1 WST-1

Al_2O_3, $CoAl_2O_4$, Fe_2O_3 and TiO_2 NPs did not induce any significant change in J774, A549 and C3A cell viability 24 post exposure, according to the WST-1 assay (Supplementary data Figure 1). Ag, QDs and ZnO NPs were the most toxic NPs tested in this study and induced a concentration dependent decrease in viability in all cell types at 24h (Figure 2). ZnO NPs were the most toxic NP tested, inducing a significant increase in cell death at concentrations greater than 5µg/cm^2 in all cell types. Ag NPs induced a significant decrease in cell viability at concentrations greater than 6.25µg/cm^2 in A549 and C3A cells. QDs caused a significant reduction in cell viability at concentrations greater than 5 µg/cm^2 in J774 cells, and at concentrations greater than 40 µg/cm^2 in A549 and C3A cells. LC_{50} values (concentration required to kill 50% of cells) were calculated for Ag, QDs and ZnO NPs in all cell types, and are represented in Table 4. Based on the LC_{50} values it was evident that macrophages are the most sensitive cell type investigated and that ZnO NPs were consistently the most toxic NP. Based on the findings from the WST-1 assay and data obtained using J774 cells, the toxicity of the NP panel can be ranked as follows: ZnO>QDs>Ag>TiO_2>Co=Fe>Al. In keeping with these results, both the C3A and A549 cells exhibited the same ranking profile for ZnO, QDs and Ag NPs.

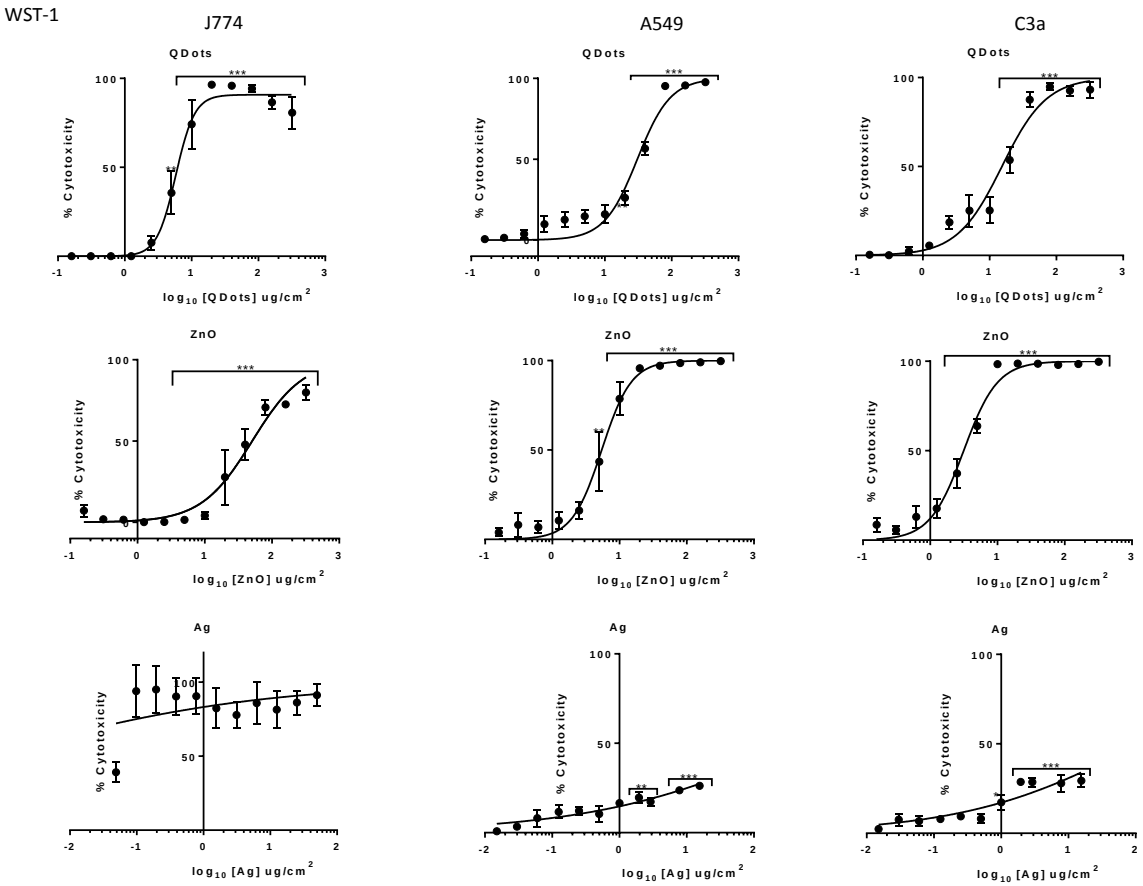

Figure 2 Cytotoxic effects of NPs on J774 mouse macrophages, A549 alveolar epithelial cells and C3A hepatocytes as determined by the WST-1 assay. The data represent the percentage cytotoxicity compared with control cells (exposed to cell culture medium). Cells were treated with NPs (0 to 320µg/cm^2, equivalent to 0 to 1000µg/ml) for 24 hours. The concentrations used for Ag NPs ranged from 0 to 50µg/cm^2 (equivalent to 0 to 16µg/ml) due to their high toxicity. Data represent the mean cytotoxicity±SEM from three separate experiments. (*** p<0.001; ** p<0.01; * p<0.05).

3.2.2 LDH assay

Al_2O_3, $CoAl_2O_4$, Fe_2O_3 and TiO_2 NPs did not induce any significant change in J774, A549 and C3A cell death, assessed via measurement of LDH release (Supplementary Material Figure 2). Ag, QDs, ZnO NPs were the most toxic NPs tested and induced a concentration dependent increase in LDH release, which is indicative of cell death (Figure 3). As observed for the WST-1 assay (Figure 2), increasing concentrations of

Ag, QDs and ZnO NPs stimulated a concentration dependent increase in cytotoxicity. QDs were the most toxic NP tested, and induced a significant decrease in cell viability at concentrations greater than 2.5µg/cm^2 in all cell types. ZnO NPs stimulated a significant increase in LDH release at concentrations greater than 5µg/cm^2 in J774 cells, 40µg/cm^2 in A549 cells and 20µg/cm^2 in C3a cells. Ag NPs were relatively non-toxic, with a significant increase in cell death observed only at the highest concentration tested (16µg/cm^2) in C3A cells. Ag, ZnO NPs and QDs and were demonstrated to be the most toxic, when the LDH assay was used to assess toxicity.

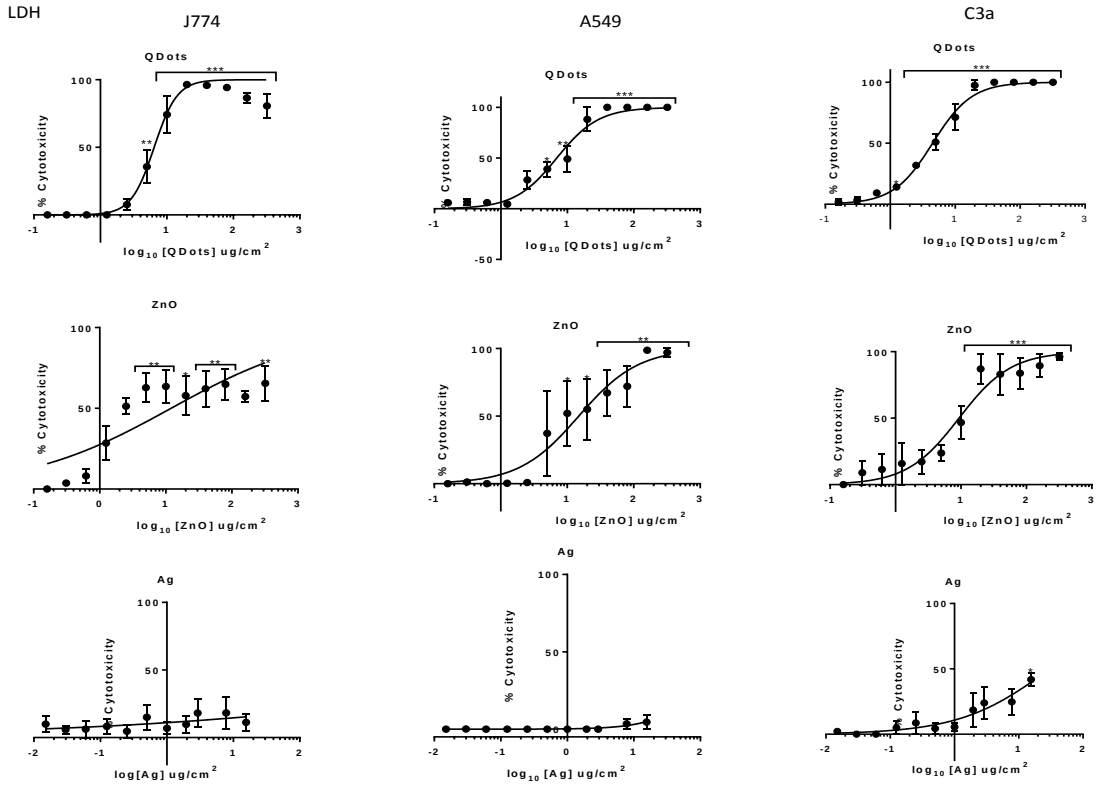

Figure 3 Cytotoxic effects of NPs on J774 mouse macrophages, A549 epithelial cells and C3a hepatocytes as determined by the LDH assay. The data represent the percentage cytotoxicity compared with triton exposed cells. Cells were treated with NPs (0 to 320µg/cm^2 equivalent to 0 to 1000µg/ml) for 24 hours (ZnO and QDots). The doses used for the Ag particles ranged from 0 to 50µg/cm^2 equivalent to 0 to 16µg/ml) due to their high toxicity. Results are expressed as a percentage of 100% lysis using triton. Data represent the mean±SEM from three separate experiments. (*** $p<0.001$; ** $p<0.01$; * $p<0.05$).

Figure 4 summarises and compares the LC_{50} values from the WST-1 and LDH assays of all NPs across all 3 cell types. The relatively high toxicity of ZnO, Ag and QDs (demonstrated by their lower LC_{50} values), and the lower toxicity of Fe_2O_3, $CoAl_2O_4$ and TiO_2 (due to their higher LC_{50} values) is evident across all cell types. Across the three cell types, the ranking of NP toxicity was ZnO>QDots>Ag. We did not detect any interference of the NPs in either assay (data not shown). J774 cells were the most sensitive cell type to NP toxicity. The data also clearly demonstrate the higher sensitivity of the WST-1 assay (compared to the LDH assay).

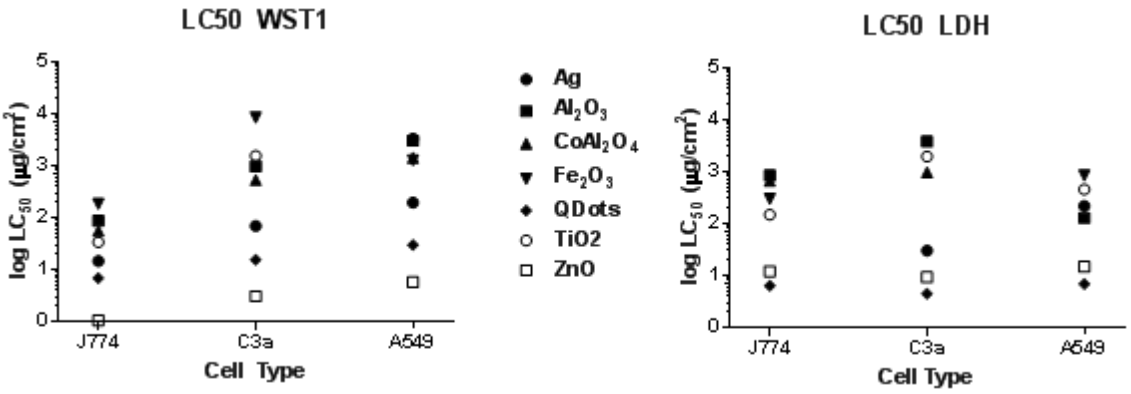

Figure 4 Scatterplots of LC_{50} values of each NP type after treatment of the different cell types (J774 macrophages, C3a hepatocytes and A549 alveolar epithelial cells) for 24 hours using the WST-1 and LDH assays.

3.3 Cytotoxic effects of Zn and Ag salts on J774 cells

The cytotoxic effects of aqueous solutions of Zn and Ag on J774 cells using the WST-1 and LDH assays are shown in supplementary figure 3 and suggest that part of the toxic effects of ZnO and Ag NPs is due to soluble ions. As indicated in this figure, it was not possible to calculate an LC_{50} value for these data.

3.4 *In vivo* Studies

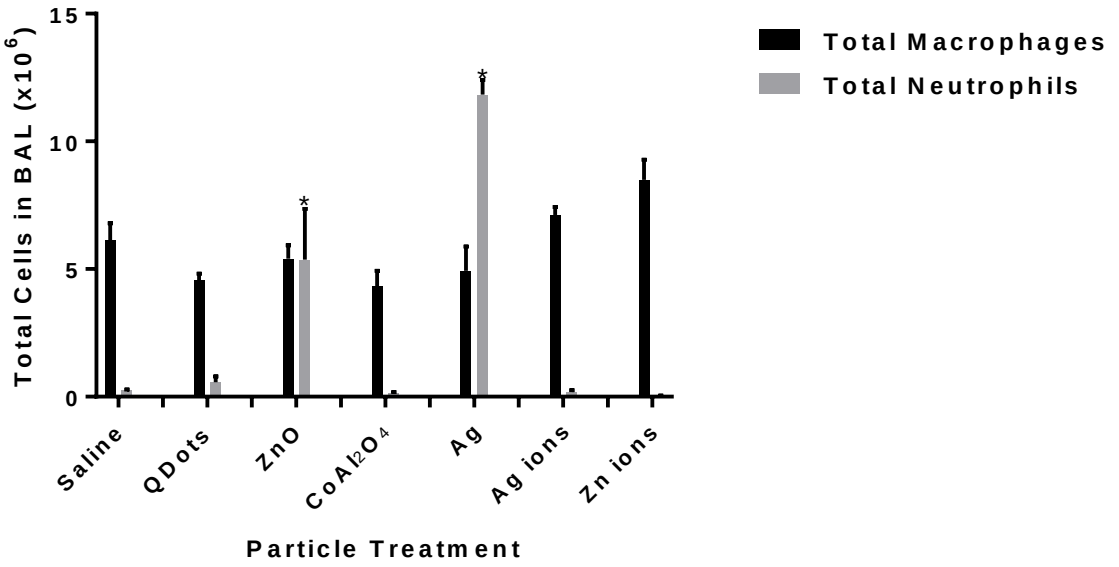

Figure 5. Total number of macrophages and neutrophils ($x10^6$) in the BAL from rats instilled intratracheally with 62.5µg of ZnO, Ag, QDs, Al_2O_3, $CoAl_2O_4$, Fe_2O_3 or TiO_2NPs or an equivalent metal ion concentration of Ag and Zn. Animals were sacrificed 24 hours post treatment, and differential cell counts performed. Data represent the mean+SEM of the total number of cells obtained from the lungs of three animals. Significance is indicated by *: $p<0.05$.

3.4.1 Total cell counts

The pulmonary inflammatory response stimulated by NPs in rats was evaluated 24 h post exposure (Figure 5). QDs, Ag, and ZnO NMs were prioritised for investigation due to their high toxicity, as observed *in vitro* and in the environmental models. Al_2O_3, $CoAl_2O_4$, Fe_2O_3 and TiO_2 NPs all exhibited low toxicity in the *in vitro* (mammalian cell and algae) and invertebrate (daphnia and worm) models. To minimise the use of animals in the study only one low toxicity NP was investigated. There were significantly more neutrophils ($p<0.05$) in the BAL of rats instilled with Ag and ZnO particles compared with the control suggesting that these two particle types were more inflammogenic (Figure 5). No neutrophil response was stimulated by QDs, and $CoAl_2O_4$ NPs or the metal salts (Ag and Zn). There was no change in macrophage numbers for any of the NPs tested 24h post exposure, compared to the control (Figure 5).

3.5 Ecotoxicology Studies

A large amount of data were collected when assessing the aquatic toxicity of the NP panel; seven NPs were tested in three organisms at various NP concentrations and time points. For all NPs at least one repetition of experiments for all organisms was performed. However, it was not possible to perform three replicates of each experiment for each NP, due to time constraints. Therefore the findings obtained from the first replicate of each experiment, combined information on NP toxicity obtained from the *in vitro* studies conducted with mammalian cells was used to prioritise the selection of NPs for more in depth toxicity testing (i.e. performing more replicates in ecotoxicity studies). Ag, QDs and ZnO NPs were observed to be consistently toxic across all models, and so only data obtained for these NPs are presented. Al_2O_3, $CoAl_2O_4$, Fe_2O_3 and TiO_2 NPs did not induce any significant changes in viability in all organisms tested. In order to summarise data obtained from all organisms and particles LC_{50} values have been calculated and are presented in Figure 6 and Table 4.

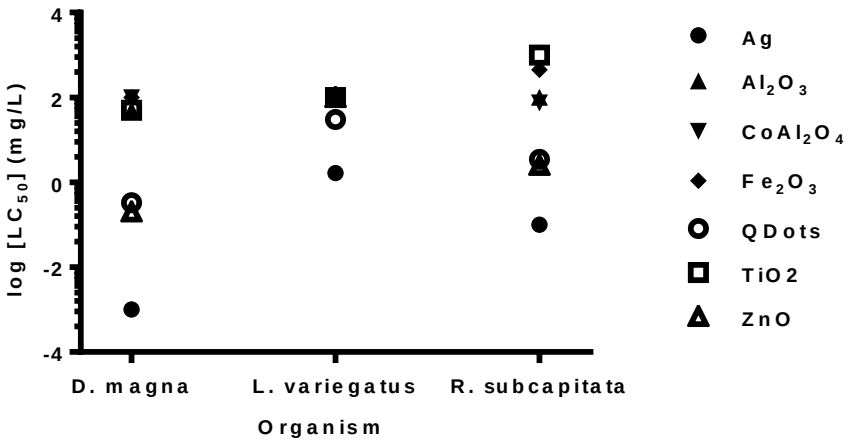

Figure 6 Comparison of LC_{50} values of each particle type after treatment of the different aquatic organisms used in this study.

3.5.1 D. magna

Ag NPs were the most toxic NP tested to *D. magna* neonates (Figure 7). A dose concentration dependent increase in Ag NP toxicity (using immobilisation as an endpoint) was observed at both 24 hours and 48 hours. The LC_{50} concentration for

Ag NPs at 24 hours was 0.0163mg/l compared with 0.0022mg/l at 48 hours Toxicity was significantly ($p<0.001$) increased at 48 h compared with 24 h.

QDs were less toxic to *D. magna* than Ag NPs, and exhibited a concentration dependent increase in toxicity at 48 hours ($p<0.05$). The LC_{50} concentration at 24 hours was not measurable, but at 48 hours, this value was 0.0004mg/l. There was no significant difference between the toxicity at 24 hours compared with that at 48 hours

ZnO was the least toxic of these three NPs but also induced a concentration dependent increase in toxicity. The LC_{50} concentration at 24 hours was 0.27mg/l and 0.199mg/l at 48 hours. Toxicity was significantly ($p<0.001$) increased at 48 h compared with 24 h.

The effect of the remaining particle types are shown in supplementary data Figure 4.

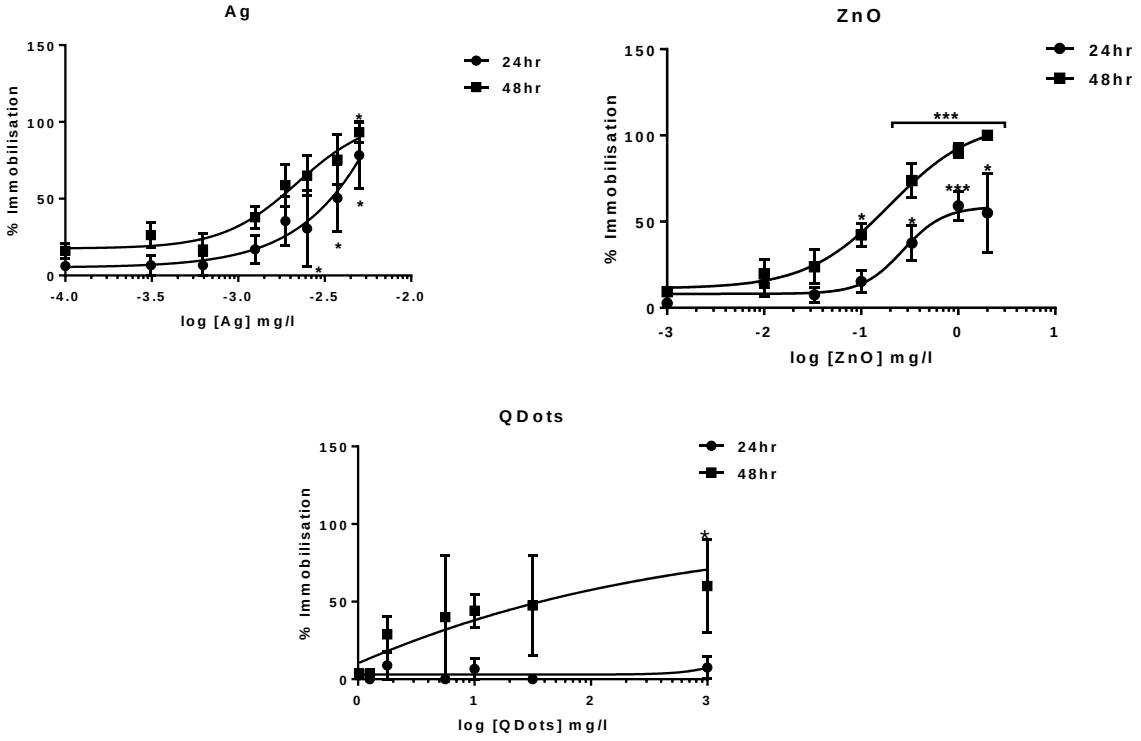

Figure 7 The effect of Ag, ZnO and QDots after 24 and 48 h exposure in *Daphnia magna*. Data represent the percentage of immobilisation of the organisms after treatment with Ag, ZnO and QDots post 24 and 48 hours exposure (two replicated experiments with three replicates each). Error bars represent the SEM * $p<0.05$ ** $p<0.01$ and *** $p<0.001$ compared with the lowest dose of particles. There was a

significant dose response for each particle type at both time points except in the case of QDots at 24 hours exposure.

3.5.2 L. variegatus

There was no significant dose effect of ZnO, Ag or QDots on *L. variegatus* mortality 96 hours post exposure (data not shown). However, the data suggest that Ag NPs were the most toxic to *L. variegatus* out of the panel of particles tested, although no significant changes in *L. variegatus* viability were observed.

Al_2O_3 $CoAl_2O_4$, Fe_2O_3 and TiO_2 did not induce mortality or changes in behaviour at the concentrations tested (data not shown).

3.5.3 R. subcapitata

Ag NPs were the most toxic NP (out of the panel tested), and induced a significant inhibition in *R. subcapitata* growth at concentrations >0.08 mg/l ($p<0.01$) (Figure 8). Above this concentration, growth was significantly inhibited ($p<0.001$). The greatest effect was evident at 24 h, with improvements in algal growth evident at 48 h and 72 h, indicating an algal compensation mechanism or detoxification of the NPs or ions in the medium over time.

Quantum dots were moderately toxic to *R. subcapitata*. As for the Ag NPs, the toxicity increased dramatically within a narrow concentration range, with a significant increase in toxicity evident at concentrations >2.0 mg/L ($p<0.001$) compared with the lowest dose. Toxicity was greatest at 24 hours.

ZnO caused no growth inhibition at doses of up to 0.3 mg/L. Growth inhibition first appeared in some of the 1 mg/L samples after 24 h, but growth had returned to control levels at 48 and 72 h.

No significant impact on algal growth inhibition was observed for Al_2O_3, $CoAl_2O_4$, TiO_2, and Fe_2O_3 (supplementary material Figure 5).

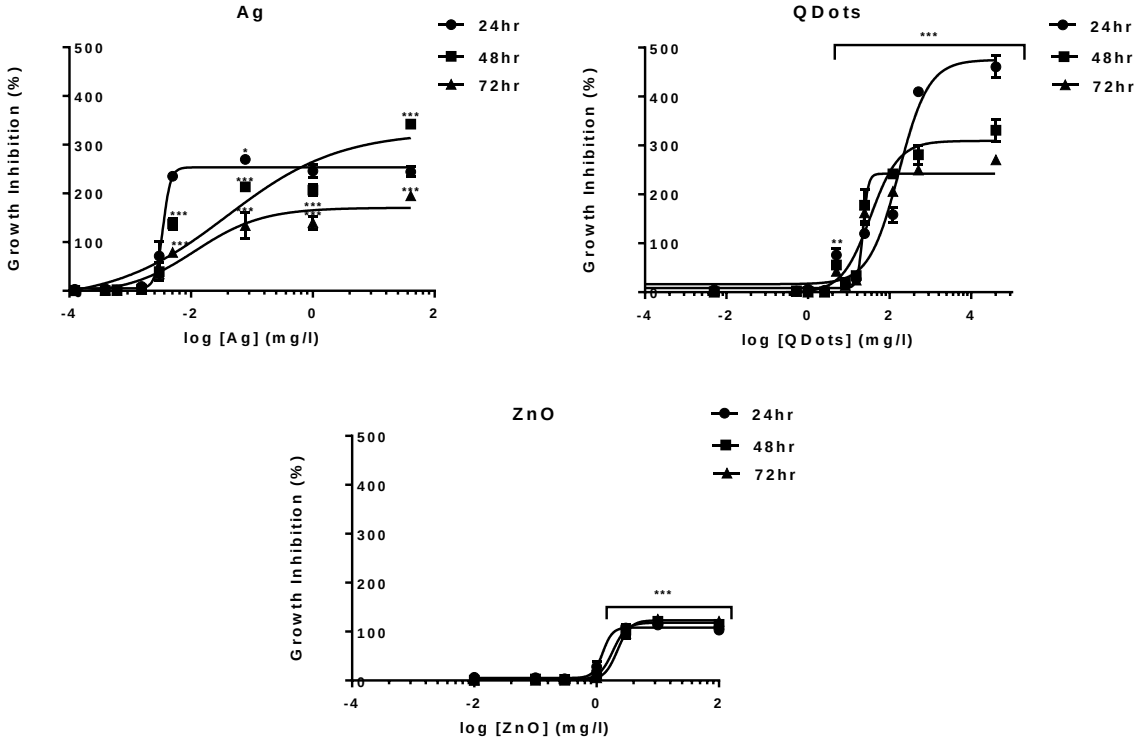

Figure 8 The effect of Ag, ZnO and QDots on algae *R. subcapitata* post 24, 48 and 72 hours exposure. Data represent the percentage growth inhibition over these time points.

3.6 Comparison of NP toxicity across models

The LC_{50} values for all NPs across all models are presented in Figure 4, and Table 2. Using the LC_{50} values (Table 2), the sensitivity of each model was compared. As shown in table 1, mammalian cell sensitivity can be ranked as J774> C3A=A549. Environmental species sensitivity, can be ranked as: *D. magna* > *R. subcapitata* > *L. variegatus.* NP toxicity can be ranked as ZnO>QDs>Ag for mammalian cells, and for Ag>ZnO>QDs in the environmental models.

Table 1 Ranking of NP (ZnO, Ag and QDs) toxicity across all models. Most toxic NPs are ranked as 1 (black), and least toxic as 3 (light grey).

	Macrophage J774	Hepatocyte C3a	Lung A549	Rat	*R. subcapitata*	*D. magna*	*L. variegatus*
ZnO	1	1	1	2	2	2	3
Ag	3	3	3	1	1	1	1
QDs	2	2	2	3	3	3	2

Table 2 LC_{50} values for each NP across all models. LC_{50} values are expressed as µg/cm^2 and µg/ml for the cell exposures and mg/L for the environmental organism exposures, and were calculated using a four parameter logistic curve for Ag, ZnO and QDots NPs (WST-1 data were used for the calculation of LC_{50} for cells, as this assay was more sensitive than the LDH assay).

	Macrophage J774	Hepatocyte C3a	Lung A549	*R. subcapitata*	*D. magna*	*L. variegatus*
ZnO	1.06 µg/cm^2 (3.31 mg/L)	3.1µg/cm^2 (9.69 mg/L)	5.77µg/cm^2 (18.03 mg/L)	2.5 mg/L	0.2mg/L	100mg/L
Ag	14.81µg/cm^2 (46.28 mg/L)	70µg/cm^2 (218.75 mg/L)	196µg/cm^2 (612.5 mg/L)	0.1 mg/L	0.001mg/L	1.65mg/L
QDs	6.91µg/cm^2 (21.59 mg/L)	15.68µg/cm^2 (49.0 mg/L)	30µg/cm^2 (93.75 mg/L)	3.5 mg/L	0.33mg/L	30mg/L

4. Discussion

This study aimed to compare the toxicity of NPs that are used by the pigment and ink industry, to identify potentially hazardous NPs whose surface will be modified in follow on studies in order to reduce their toxicity. A cross-species comparison revealed that a very similar pattern of NP toxicity was observed across mammalian (*in vitro* and *in vivo* (rodent)) and aquatic ecotoxicological models. ZnO, Ag and QDs were consistently demonstrated to be the most toxic NPs across the cross-species battery of tests employed in the study. The testing strategy used allowed identification of sensitive models (J774 cells and *Daphnia magna*) and endpoints (cytotoxicity (WST-1 assay) and immobility/lethality respectively) to screen NP toxicity in the future. The information obtained from this study should be exploited when implementing streamlined, alternative, and intelligent testing strategies to assess NP toxicity in the future. In particular, we suggest that these alternative models can be used for screening purposes when comparing the toxicity of a panel of NPs, with the data obtained used to rank the toxic potency of the NPs, which can be used for decision making. More specifically, a comparison of the toxic potency of a panel of NPs can

support the selection of low toxicity NPs for use in products, whereas the identification of higher toxicity NPs may suggest that more comprehensive hazard tests are employed to provide a more in depth understanding of their toxicity. As our study observed that *in vitro* and invertebrate (daphnia) models provide a relatively good prediction of *in vivo* toxicity, our testing approach will mean that not all NPs need to be tested in rodent models, which is likely to minimise animal use in nanotoxicology. In addition the results obtained from alternative models could be used to help refine the design of focused animal studies (e.g. by avoiding testing high toxicity NPs whose administration could lead to pain and suffering, or to support dose selection for rodent studies to refine their experimental design). As our approach to NP toxicity testing can reduce the burden placed on rodent testing and promote the use of *in vitro* and invertebrate models as alternatives to rodents, we provide an opportunity to better align nanotoxicology testing with the 3Rs principles (Burden *et al*., 2016).

4.1 Sensitivity of test systems

This study used three mammalian cell types representing the lung, immune system and liver to investigate the impacts of NPs *in vitro*. The toxicity of the NPs *in vivo*, was also assessed in the rat lung, following exposure via intratracheal instillation. In addition the aquatic toxicity of NPs was assessed in three environmental model organisms representing primary producers and invertebrates (including organisms that inhabit the water column and sediment). On testing the toxicity of a panel of 7 NPs, of varied physico-chemical properties in each model, it was consistently demonstrated that Ag, QDots and ZnO NPs were of higher toxicity than the other NPs tested in the study. These findings align with existing mammalian and environmental studies which frequently demonstrate the high toxicity of Ag, QDs and ZnO compared to other NP types (e.g. Johnston *et al*., 2015, Kermanidazeh *et al*., 2013, Mallevre *et al*., 2014, Zhang *et al*., 2015, Song *et al*., 2014, Gosens *et al*., 2015, George *et al*., 2011).

J774 cells were the most sensitive cell type investigated in the *in vitro* component of this study. J774 cells were selected to investigate the macrophage response to the NP panel. Macrophages are phagocytic cells, which are specialised to recognise and remove foreign material following exposure, and are known to accumulate NPs *in vivo* and *in vitro*. Activation of macrophages is a common phenomenon associated with

particle exposure (e.g. Brown *et al*., 2004), and whilst the activation of inflammatory responses to foreign material (such as NPs) is a normal, physiological, protective response the stimulation of persistent inflammation can lead to adverse health effects. This study focused on investigation of cytotoxicity, in order to rank the toxicity of the NP panel and future studies should assess sub-lethal impacts on macrophage function (e.g. cytokine production). We standardised the concentration of NPs for each cell type investigated as ug/cm^2. However, differences in sensitivity between the cells/organisms in our study could be attributed to differences in dosimetry and more specifically the particle dose delivered to the cells/organisms in each test system (i.e. mammalian cells, algae, daphnia, worms). Therefore, investigation of NP dosimetry in different test systems which require the preparation of NPs in aqueous media would assist in the interpretation of the hazard data obtained in this study. Indeed, mathematical models have been developed that enable a estimation of NP dosimetry for *in vitro* systems (e.g. Teeguarden et al., 2007, Cohen et al., 2013 and 2014, DeLoid et al., 2014, Pal et al., 2015). Such models have been used to investigate the relationship between NP dosimetry and the toxicity of NPs *in vitro* (e.g. Cohen et al., 2013 and 2014, DeLoid et al, 2014). However existing mathematical models that have been designed to predict NP-cell interactions assume that the test biological system (e.g. cell) is on the bottom of the test vessel this is not appropriate for environmental organisms such as daphnia and worms which are mobile organisms. Furthermore, the models assume static conditions which is not relevant for the algal experiments conducted in our study. In addition, we did not prepare the NPs according to the recommended dispersion protocols developed for such dosimetry estimates. Therefore, whilst we acknowledge that it is important to estimate NP dosimetry in each test system it was not considered appropriate to apply existing dosimetry models to our study. However, the results from existing studies (e.g. Cohen et al., 2013, Pal et al., 2015) suggest that differences in the hazard ranking between NPs in our study may derive from differences in the dose that is delivered to cells / organisms (i.e. dosimetry). Accordingly, it is recommended that these mathematical models are adapted and extended in the future in order to help better understand NP dosimetry in different test systems to support a more robust ranking of NP toxicity across different models.

Of interest is that ZnO NPs was always identified as the most toxic NP tested in *in vitro* cell models in this study. This is in accordance with existing *in vivo* research which has demonstrated the relatively higher toxicity of ZnO NPs compared to Ag NPs (e.g. Gosens *et al*., 2015, Song *et al*., 2014). Our *in vivo* study demonstrated that the toxicity of ZnO and Ag NPs was comparable. Particle size and of the release of Ag^{+} and Zn^{+2} ions from Ag and ZnO NPs through their dissolution, is hypothesised to play an important role in their toxic effect. Hatipoğlu *et al*., (2015) demonstrated that, in addition to the released ions, incomplete nuclei AgNPs (a few nm of AgNPs) during the AgNP synthesis can contribute to the observed toxicity. As seen from TEM image of AgNPs(Figure 1) and DLS measurements (supplementary data tables 2 and 3), the AgNPs are quite polydisperse and their size ranges from 2 nm to approximately 30nm, which may contribute to their toxicity. The solubility of NPs is known to be influenced by pH, with NP dissolution enhanced at low (acidic) pH (e.g. Cho *et. al.* 2012). This may explain why macrophages were the most sensitive cell type to NP toxicity in our study, as it is likely that NPs were internalised by macrophages via phagocytosis and delivered to lysosomes where the acidic pH of this organelle may encourage NP dissolution. One of the limitations of working with static, *in vitro* models is that there is no movement of material, and thus soluble components are in contact with cells for longer than would likely occur *in vivo* which could enhance the toxicity of soluble NPs *in vitro*.

Daphnia were the most sensitive aquatic organism investigated in this study. This is consistent with existing ecotoxicity studies (Gaiser *et al*., 2011; Kalman *et al*., 2015, Khan *et al*., 2015; Sohn *et al*., 2015). Previous studies have also demonstrated that different environmental organisms vary in their susceptibility to NP toxicity. For example, Sohn *et al*., (2015) ranked the order of animal susceptibility to Ag NPs as *Daphnia*>algae>fish. This finding that *Daphnia* are the most sensitive species to NP toxicity aligns with the findings of our study.

There is a large diversity of NPs whose safety needs to be assessed, across their value chain and life cycle. There are many mammalian (*in vitro, in chemico* and *in vivo*) and environmental models that can be used to assess NP toxicity; ranging from assessment of NP reactivity in acellular assays, investigation of the interaction of NPs with biological molecules (e.g. proteins, DNA) in in chemico assays, as well as the use of fractions of cells, algal cells, bacterial cells, mammalian cells (primary or cell line),

and whole organisms (invertebrates (e.g. daphnia, worms) and vertebrates (e.g. fish, rodents)). It will not be possible to assess the safety of NPs in all available models due to time, and cost considerations. In addition, it is prudent to encourage the use of alternative models that do not require the use of vertebrates (e.g. rodents) as this is more ethically responsible, cheaper and quicker. An increased implementation of alternative models to assess NP toxicity would better align nanotoxicology studies to the 3Rs principles (Burden *et al*., 2016). Data obtained from this study have identified that J774 macrophages were the most sensitive mammalian cell model tested, and that *D. magna* were the most sensitive aquatic environmental organism. Therefore we suggest that the use of these models is prioritised when screening the toxicity of NPs in the future. Of benefit, is that the data obtained from the cell and daphnia models align with the rodent data. Accordingly, the findings also have the potential to reduce the requirement for rodent testing. Therefore, the use of invertebrates such as *Daphnia magna*, may allow for the testing of NPs in whole (invertebrate) organisms to reduce or replace animal (rodent) testing in the future.

4.2 Selection of endpoints

Assessment of cytotoxicity is a valuable tool when screening NP toxicity *in vitro*, as it enables the identification of indicators of toxicity (e.g. LC50) that can be used to compare NP toxicity and identify sub-lethal NP concentrations to test when assessing the mechanism underlying any observed toxicity. There are multiple assays that can be used to assess the ability of NPs to impact on cell viability (e.g. WST-1, Alamar Blue, Neutral Red, LDH, MTT assays). Of benefit is that the WST-1 and LDH assays can be performed in one experiment as the cells can be used for the WST-1 assay, and the cell supernatant for LDH activity analysis (to measure LDH release/leakage due to a lack of membrane integrity as an indicator of cell death). This study demonstrated that the WST-1 assay was more sensitive than the LDH assay. This result is to be expected, as the WST-1 assay measures mitochondrial activity, which is likely to precede membrane damage which is measured in the LDH assay. The finding that the LDH (release) assay is less sensitive than assays which assess metabolic function (e.g. alamar blue, WST-1) also aligns with existing studies which have investigated NP toxicity *in vitro* (e.g. Gliga *et al*., 2014; Soares *et al*., 2016). There are an array of cytotoxicity/viability assays available to assess NP toxicity *in vitro*, and the choice of assay used by investigators is frequently based on expertise, availability

of equipment, and to fulfil the testing of specific hypotheses (e.g. when investigating the mechanism underlying NP toxicity) as well as cost, and time considerations. Whilst the MTT assay (which assesses mitochondrial function as a marker of cell viability) was previously a popular assay used to assess particle induced cytotoxicity, evidence of NP interference with the MTT assay (Worle-Knirsch *et al*., 2006), led to a reduction in its use. Such findings have encouraged the routine assessment of NP interference when investigating NP cytotoxicity, as well as for a range of other assays. Alternative cytotoxicity assays (e.g. WST-1 and Alamar blue assays) have become more popular over recent years, and assess mitochondrial function as a marker of cell viability but in contrast to the MTT assay do not require the product to be solubilised, which reduces the time taken to assess toxicity. It is recommended that researchers continue to assess NP induced cytotoxicity via at least two different assays, which assess cytotoxicity via different mechanisms. Where possible, the assays should be performed within one experiment; e.g. utilising the cells themselves (e.g. alamar blue, WST-1 assays) and the cell supernatant (e.g. LDH assay). In fact, the simultaneous application of three cytotoxicity assays in one experiment has been demonstrated recently (Connolly *et al*., 2015). Of additional benefit is that the use of different assays, may provide a better understanding of the cell and molecular mechanisms underlying NP toxicity. If only one assay can be performed, the results from this study suggest that the WST-1 assay is the most sensitive and should be prioritised.

4.3 Limitations of the study

In vitro tests assessed NP toxicity using 3 cell types, representing the lung, liver and immune cell responses. The response of other target sites was not considered. For example, models of the skin and gastrointestinal tract (GIT) were not included in the assessment, although it is anticipated that dermal exposure or ingestion of NPs produced by the pigment and ink industries is likely during their production, use and disposal. It would therefore be interesting to test the toxicity of NPs using a wider array of cell types that represent different target sites, to identify differences in cell sensitivity to NP toxicity. Macrophages reside in a number of locations (e.g. GIT, skin, lung) and are likely to contribute to the local and systemic toxicity of NPs. Of relevance is that Kermanizadeh *et al*., (2016) demonstrated the response of cells from the pulmonary, cardiovascular, hepatic, renal and developmental systems to a panel of NPs was comparable and enabled a similar ranking of toxicity to be obtained. Therefore, whilst

cells can vary in their sensitivity to NP toxicity, the overall pattern of response measured is often similar. Of benefit is that an extensive amount of information available on the macrophage response to NPs already exists which can be used to compare the toxicity of NPs to existing data for particles of known toxicity (e.g. PM_{10}, quartz, asbestos). Cell lines were used in this study, and thus the response of primary cells could be considered in the future to identify if the findings obtained from cell lines are relevant. Existing research has demonstrated that J774 cells respond very similarly to primary human monocytes (Brown *et al*., 2004). Simple monocultures of cells were used to investigate NP toxicity in this study. More complex *in vitro* models are available which better mimic *in vivo* cell architecture, and the response of such models could be compared to the findings obtained from monocultures of cells. For example, a 3D triple co-culture of the lung, consisting of epithelial cells, macrophages and dendritic cells has been developed (Rothen-Rutishauser *et al*., 2005) and could be used in future studies. *In vitro* models are often criticised for their relevance to the *in vivo* situation. The toxicity of the NP panel was also been tested *in vivo* in our study, allowing *in vitro in vivo* extrapolations (IVIVE) to be performed to allow a better understanding of the benefits and limitations of the models used. The NP types identified as inflammogenic, based on the number of neutrophils in the lung, were ranked as Ag>ZnO>QDots=$CoAl_2O_4$). This ranking was similar to the profile shown for the toxicity of the different particle types *in vitro* (ZnO>QDots>Ag>TiO_2> $CoAl_2O_4$). Both Ag and ZnO NPs significantly increased the total neutrophil numbers in the lung and were identified as the most hazardous NPs in our study. Information in the existing literature on the *in vivo* toxicity of NPs may also be used for IVIVE analysis (e.g. Gosens *et al*., 2015).

When assessing NP safety it is important to understand the mode of action of NP toxicity. Existing evidence suggests that NPs are able to stimulate inflammatory and oxidant driven responses in cells which can lead to genotoxicity and cytotoxicity. Studies have been conducted to assess the sub-lethal toxicity of NP panel tested in this study via evaluation of cytokine production, oxidative stress (antioxidant depletion, ROS production) and genotoxicity (Brown et al., manuscript in preparation).

NPs are likely to deposit in sediments following release into the aquatic environment, due to their tendency to agglomerate/aggregate. Therefore there is a need to test NP toxicity in sediment dwelling organisms (Li *et al.,* 2014). Although *Lumbriculus* can

reside in sediment, exposure of this organism to NPs via sediment was not tested in this study. Future studies could therefore investigate impacts of NPs on terrestrial species.

Due to the large number of NPs, and organisms that were to be tested in this study, not all ecotoxicology experiments were repeated on three separate occasions. Instead, the findings from mammalian studies, and the first replicate of ecotoxiology studies were used to prioritise the selection of NP toxicity testing. This enabled NPs of most concern to undergo a more comprehensive testing of their toxicity, whilst still obtaining information on all NPs. This type of tiered and intelligent testing strategy proved beneficial and is recommended for future studies.

The dose range chosen was expansive in order to include relevant exposure concentrations and to allow LC_{50} values to be calculated for comparison and ranking purposes. The relevance of all concentrations to all NPs used to real-life exposure requires a better understanding of exposure levels (which is currently lacking). Once real-life exposure concentrations are available the dose-response data obtained in this study could provide a useful tool to assess hazard at relevant concentrations in addition to the ranking already provided.

4.4 Conclusion

This study identified potentially hazardous NPs that are used by the pigment and ink industry. A cross-species comparison revealed that Ag, QD and ZnO NPs were consistently more toxic than the other NPs tested. Thus, these NPs were selected for surface modification to reduce their toxicity (Brown *et al*., *manuscript in preparation*). By looking across mammalian and ecotoxicological models we obtained a better understanding of the toxic potency of each NP, the sensitivity of each model, and thus which models should be prioritised for selection in the future when assessing the mammalian and aquatic toxicity of NPs. The implementation of intelligent testing strategies for assessment of NP hazard which encourage the implementation of alternative models is essential and data from this study can feed into the design of a tiered testing strategy for NPs.

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Conflict of Interest

We declare that there are no conflicts of interest associated with this work.

Supplementary Material

Supplementary Table 1 NP suppliers and size information as provided by the suppliers

Particle	Supplier	Nominal Size
Iron Oxide (Fe_2O_3)	TecStar	65nm
Titanium Dioxide (TiO_2)	TecStar	18nm
Zinc Oxide (ZnO)	TecStar	98nm

Particle Type	Hydrodynamic diameter (nm)	PDI	Zeta Potential
Ag	29.57±4.83	0.431±0.007	-46.2±0.76
CdSe/ZnS	36.33±7.26	0.329±0.005	-28.7±0.85
TiO_2	518.53±6.95	0.361±0.009	-22.2±0.245
Al_2O_3	542.86±34.98	0.524±0.005	-18±0.573
Fe_2O_3	218.5±2.41	0.181±0.001	-21.1±0.245
$CoAl_2O_4$	201.47±1.64	0.290±0.009	-16.75±0.123
ZnO	903.9±16.8	0.333±0.007	-16.85±0.450

Cobalt Aluminium Oxide ($CoAl_2O_4$)	Torrecid	31nm
Silver (Ag)	Plasmachem	10nm
Aluminum Oxide (Al_2O_3)	Plasmachem	40nm
Cadmium selenide / Zinc sulphide (CdSe/ZnS) Quantum Dots (QDs)	Plasmachem	5nm

Supplementary table 2. The Hydrodynamic diameter, PDI and Zeta potentials of NPs dispersed in ddH_2O.

Supplementary table 3. Hydrodynamic diameter and polydispersive index (PDI) of NPs in cell culture medium (MEM & 10% FCS).

	0 Time		24 Hours	
Particle type	Hydrodynamic Diameter (nm)	PDI	Hydrodynamic Diameter (nm)	PDI
Al_2O_3	73.55±4.21	0.469±0.05	104.21±15.77	0.547±0.076
TiO_2	442.07±5.86	0.369±0.007	517.53±17.69	0.405±0.015
Fe_2O_3	226.63±4.63	0.185±0.002	248.33±1.96	0.176±0.013
ZnO	728.07±36.22	0.448±0.03	481.03±4.47	0.771±0.010
Ag	23.31±0.88	0.484±0.001	27.7±0.94	0.718±0.033
QDots	39.63±9.15	0.372±0.070	195.57±24.11	0.328±0.058
$CoAl_2O_4$	136.37±0.96	0.511±0.004	165.4±3.62	0.528±0.018

Supplementary table 4: Constituent salts of reconstituted hard water (USEPA, 2002).

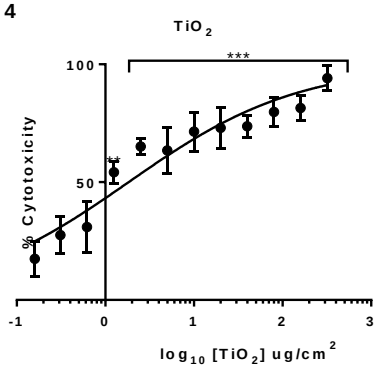
Salt	Concentration (mg/l) in DI water
Calcium sulphate dehydrate (Ca $SO_4.2H_20$)	120
Magnesium sulphate ($MGSO_4$)	120
Potassium chloride (KCl)	8
Sodium hydrogen carbonate (($NaHCO_3$)	192

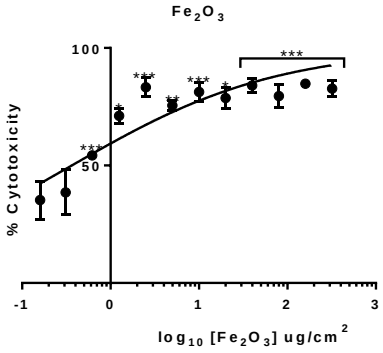
Supplementary table 5: Constituents of Jaworski's Medium

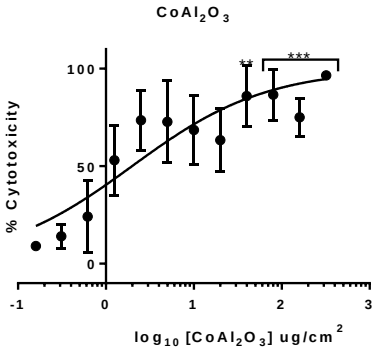
Stock	Compound	per 200ml
1	$Ca(NO_3)2.4H_2O$	4.0g
2	KH_2PO_4	2.48g
3	$MgSO_4.7H_2O$	10.0g
4	$NaHCO_3$	3.18g
5	EDTAFeNa EDTANa_2	0.45g 0.45g
6	H_3BO_3 $MnCl_2.4H_2O$ $(NH_4)6Mo_7O_{24}.4H_2O$	0.496g 0.278g 0.2g
7	Cyanocobalamin Thiamine HCl Biotin	0.008g 0.008g 0.008g
8	$NaNO_3$	16.0g
9	$Na_2HPO_4.12H_2O$	7.2g

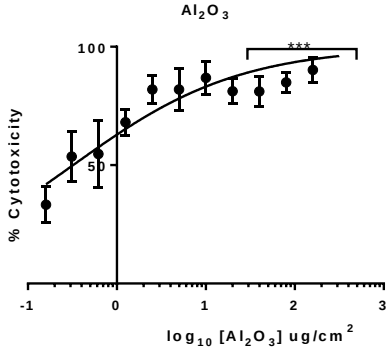
To reconstitute, add 1ml of stock solutions 1-9 to 1l deionized water.

WST-1 J774

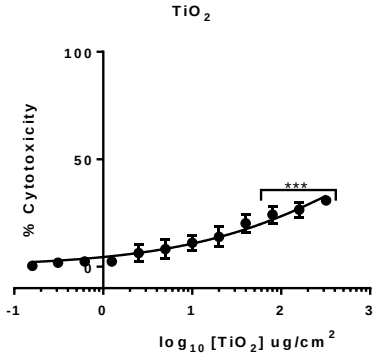


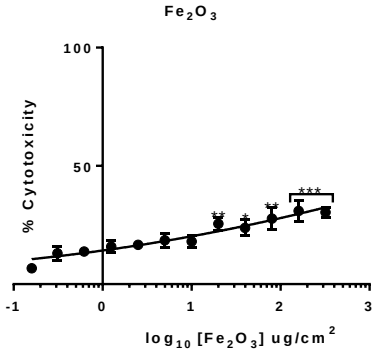


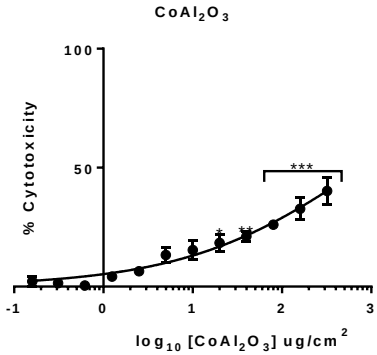


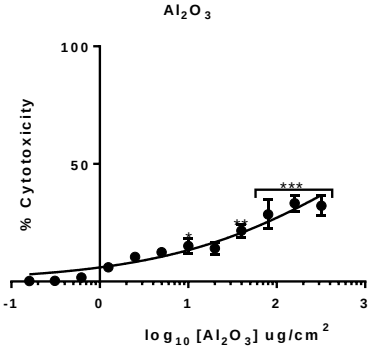


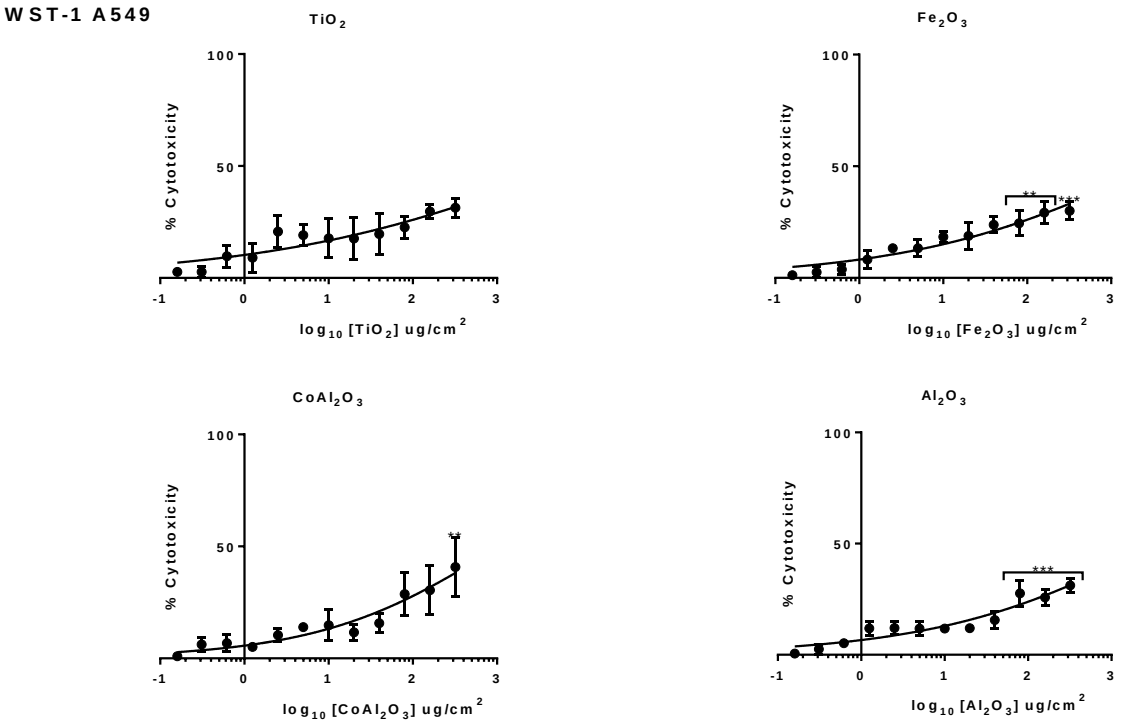
WST-1 C3a





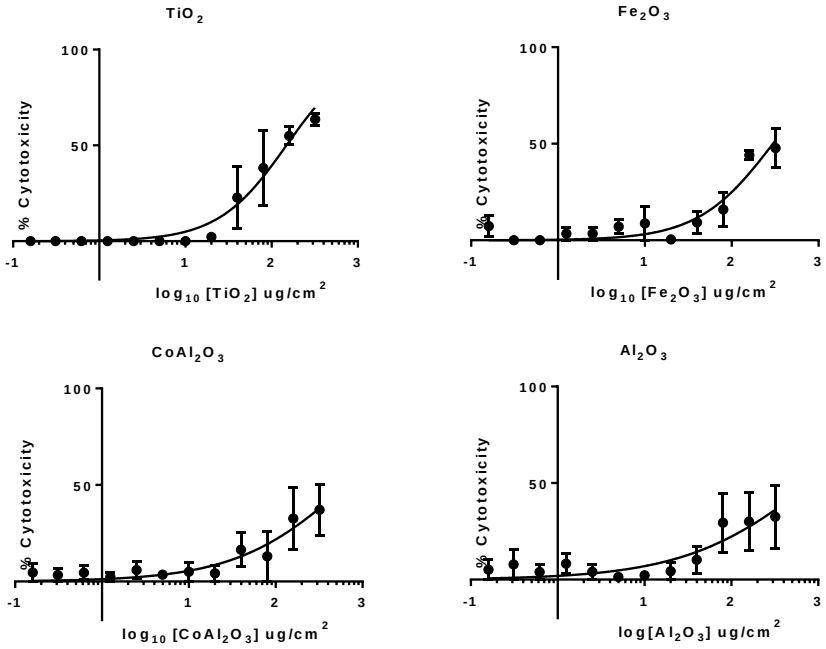






Supplementary figure 1 cytotoxic effects of nanoparticles on J774 mouse macrophages, A549 epithelial cells and C3a hepatocytes as determined by the WST-1 assay. The data represent the percentage cytotoxicity compared with untreated cells. Cells were treated with nanoparticles (0 to 320µg/cm^2) (0 to 1000µg/ml) for 24 hours. Results are expressed as a percentage of 100% lysis using triton. Data represent the mean±SEM from three separate experiments. (*** p<0.001; ** p<0.01; * p<0.05).

LDH J774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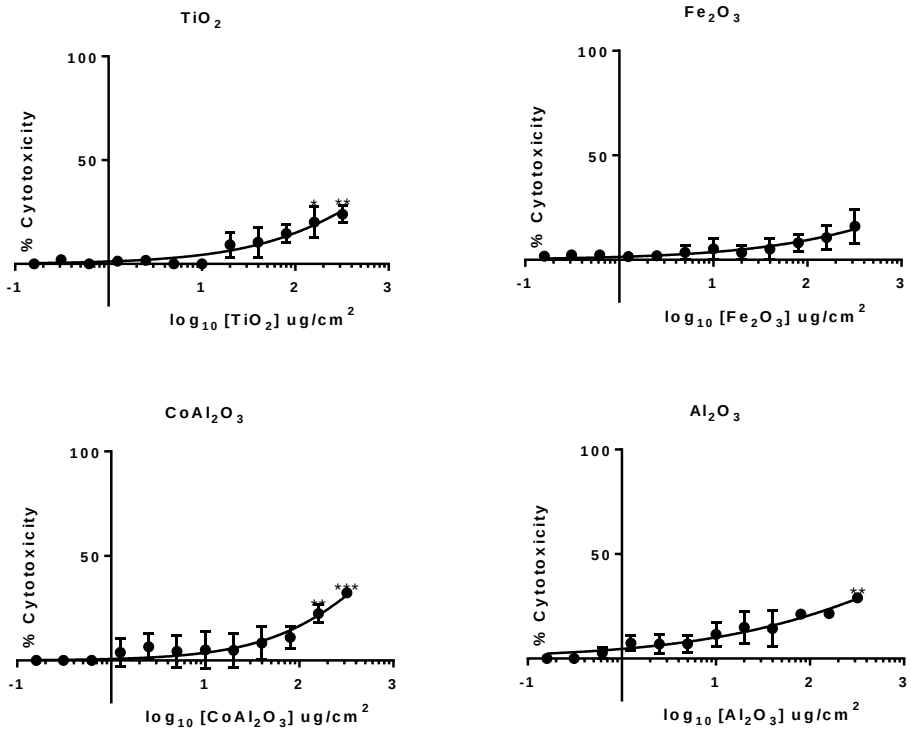
LDH C3a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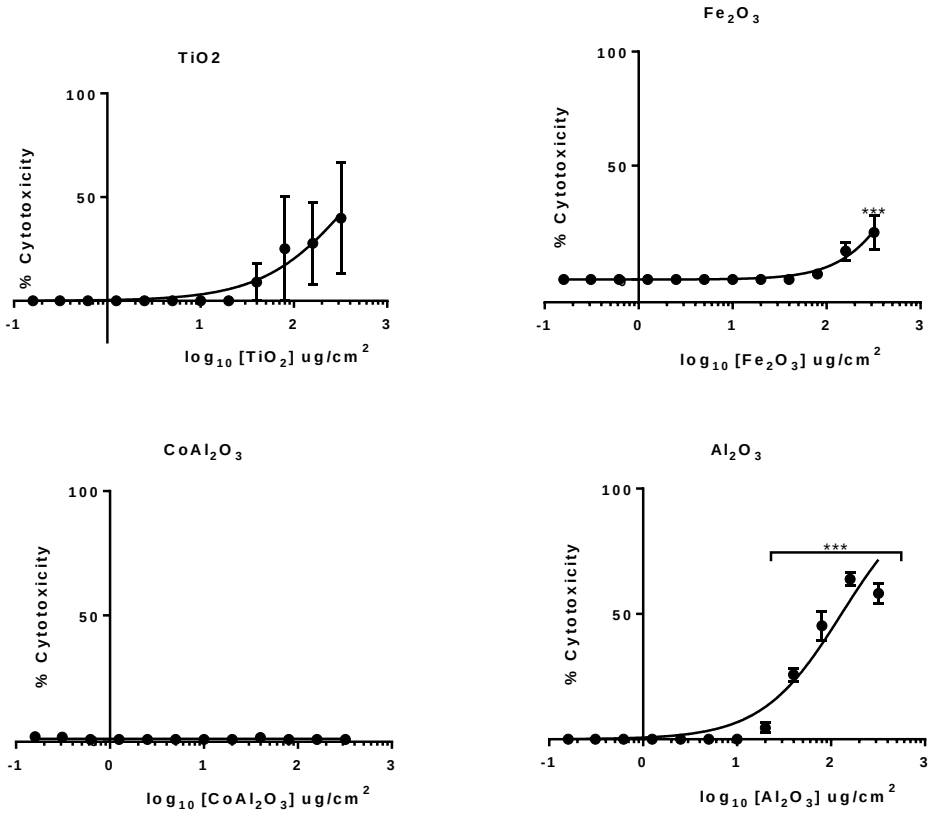
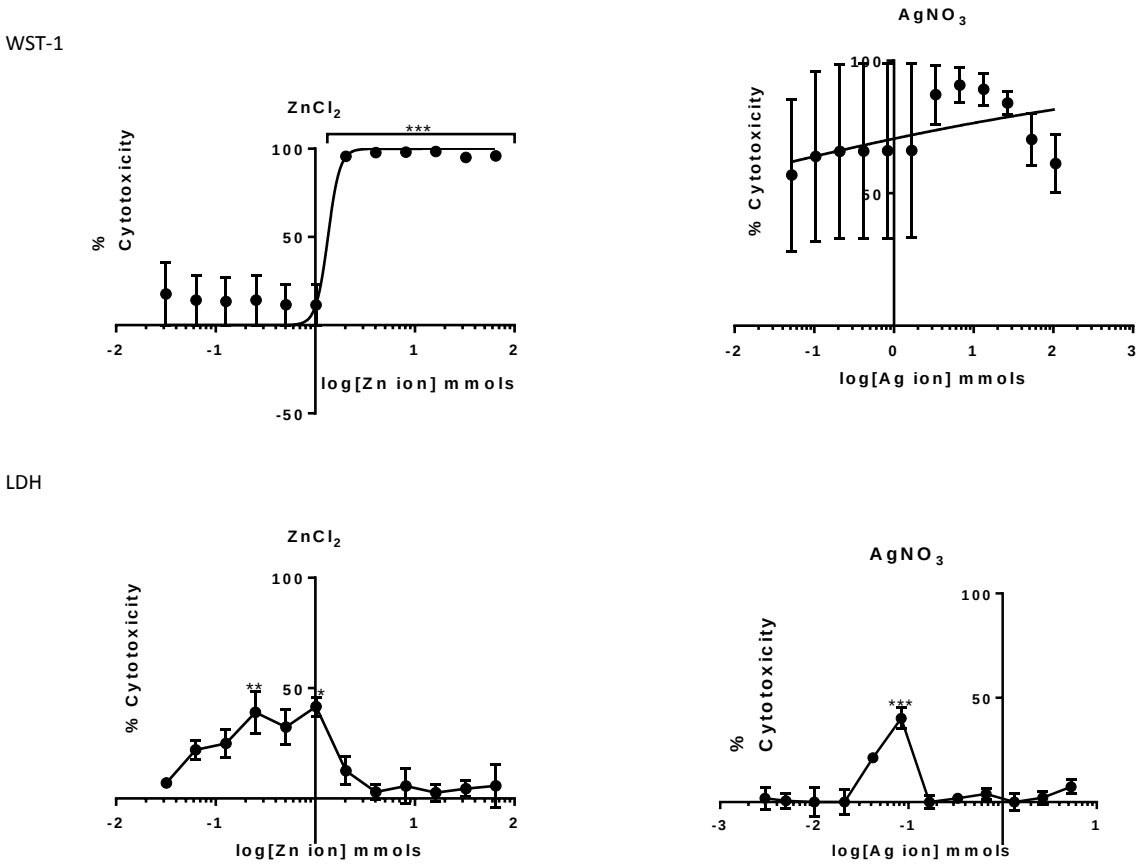
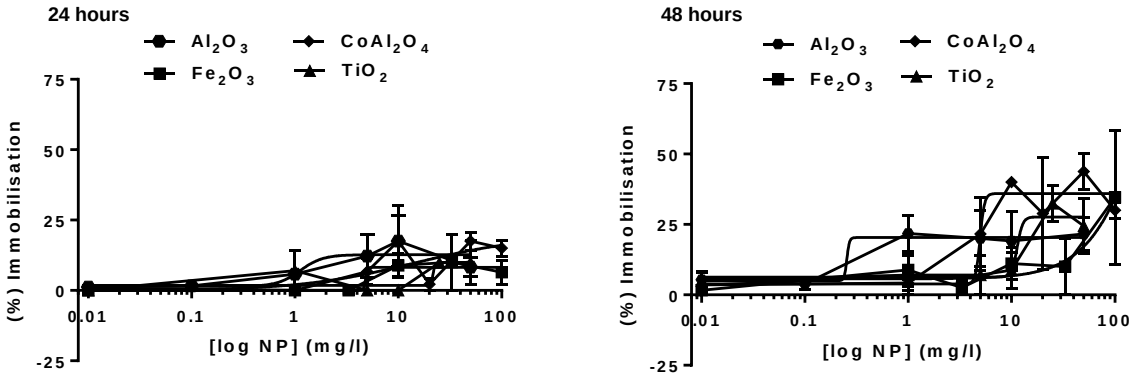
LDH A549



Supplementary figure 2 cytotoxic effects of nanoparticles on J774 mouse macrophages, A549 epithelial cells and C3a hepatocytes as determined by the LDH assay. The data represent the percentage cytotoxicity compared with untreated cells. Cells were treated with nanoparticles (0 to 320µg/cm^2) (0 to 1000µg/ml) for 24 hours. Results are expressed as a percentage of 100% lysis using triton. Data represent the mean±SEM from three separate experiments. (*** p<0.001; ** p<0.01; * p<0.05).



Supplementary figure 3 cytotoxic effects of ionic solutions of Zn and Ag on J774 mouse macrophages as determined by the WST-1 and LDH assay. The data represent the percentage cytotoxicity compared with untreated cells. Cells were treated with solutions of $ZnCl_2$ or $AgNO_3$ (0.032-65µmols Zn or 0.05-107µmols Ag) for 24 hours. Results are expressed as a percentage of 100% lysis using triton. Data represent the mean±SEM of the percentage cytotoxicity from three separate experiments. (*** p<0.001; ** p<0.01; * p<0.05 compared with untreated cells).



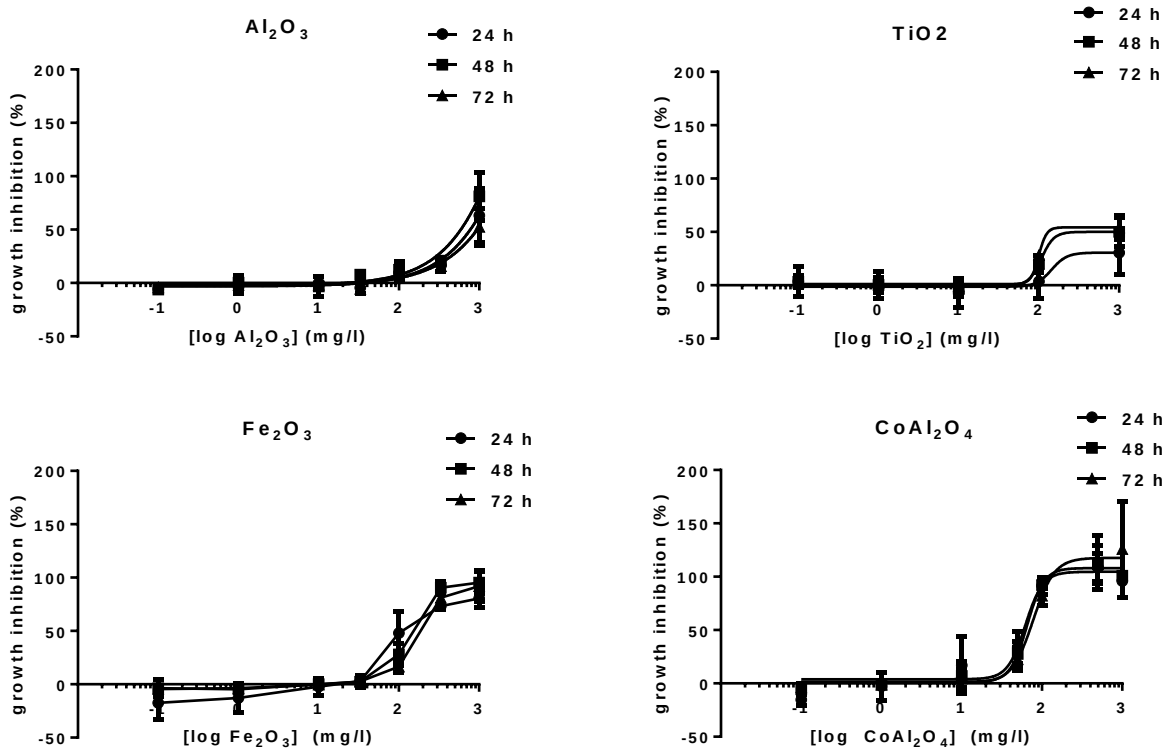
Supplementary figure 4 Effects on particles on the immobilisation of D. magna 24 and 48hours post-exposure.

Aluminium oxide NPs were used at concentrations between 0.1 and 50 mg/l. Only low toxicity was observed, which was increased at 48 h compared to 24 h, and the LC_{50} was not reached.

Toxicity of cobalt aluminate to *D. magna* was of a similar order as observed for aluminium oxide, although the toxicity appeared to plateau from around 10 mg/l. At a concentration of 20 mg/l, lower toxicity was observed; however, this concentration was only used in two of the three repeats. No dose-dependent increase in toxicity from 10 up to 100 mg/l was observed. The LC_{20} between 1 and 5 mg/l is unlikely to be reached in the environment (data not shown).

Iron (III) oxide showed very low toxicity to *D. magna*. The LC_{50} was not reached at concentrations up to 100 mg/l.

Titanium dioxide NPs, together with Fe_2O_3 particles, were the least toxic in the panel. There was no significant mortality or immobilisation of *D. magna* at concentrations of up to 50 mg/l.



Supplementary figure 5 Inhibition of growth of *R. subcapitata*. Samples were taken 0, 24, 48 and 72 hours post exposure and the optical density measured at 685nm. These values were normalised to the corresponding cell numbers and the growth inhibition calculated in relation to the control for the equivalent time point.

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